REMARKS

An Office Action was mailed in the above-captioned application on April 24, 2006. As of the Office Action, claims 67-94 were pending. Claims 67-73, 76, 80 and 82-94 were withdrawn from consideration. Claims 74, 75, 77-79 and 81 were rejected. This Request for Reconsideration document is submitted in response to said Office Action.

The Rejection under 35 U.S.C. § 102(b)

The Examiner has rejected Claims 74, 75, 77-79 and 81 under 35 U.S.C. 102(b) as being anticipated by Peltz, et al., *J. Immunol.* 141:1891-96 (1988). The Court of Appeals for the Federal Circuit has stated that anticipation requires the presence in a single prior art reference of each and every element of the claimed invention. *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1458 (Fed. Cir. 1984); *Alco Standard Corp. v. Tennessee Valley Auth.*, 1 U.S.P.Q.2d 1337, 1341 (Fed. Cir. 1986). "There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention." *Scripps Clinic v. Genentech Inc.*, 18 U.S.P.Q.2d 1001, 1010 (Fed. Cir. 1991) (citations omitted).

Specifically, the rejection states that the rejected claims are drawn to an isolated polypeptide comprising an extracellular region of a native FcγRII receptor and a fusion component, wherein the fusion component is a carbohydrate, the polypeptide is soluble, or in a pharmaceutical diluent. The rejection reasons that Peltz, et al., discloses a secreted protein containing the extracellular domain of FcγRII receptor in HEPES-saline, and that sFcγRII (26kD) is an N-linked carbohydrate.

Applicant respectfully traverses this rejection. The native N-linked carbohydrates of the of the 23kD and 26kDa forms of sFcγRII (p. 1895, left hand column, second paragraph), would not be understood by the person skilled in the art as constituting a "fusion component" in the context of the present invention. Peltz, et al., identifies the forms as "sFcγRII carrying N-linked oiligosaccharides at one and both of the two N-linked glycosylation sides *identified in the FcγRII cDNA sequence.*" Thus, in the disclosed mammalian cell expression systems, expressed sFcγRII is *naturally* glycosylated unless the cell culture is conducted in the presence of tunicamycin (p. 1892, left hand column, fourth complete paragraph).

In contrast, we consider that the term "fusion component" would be understood by the person skilled in the art as referring to something that is *foreign* to the "Fc binding component". The specification, at page 16, lines 25-35, states that augmentation of the Fc binding component "is achieved by adding a peptide,polypeptide or other molecule which is well tolerated in an animal. . . . The other molecules suitable include dextrans, carbohydrates, ..."

Additionally, reference to Example 6 of the present specification may be made. This example describes the production of a fusion protein comprising the extracellular region of FcγRII fused to human serum albumin (HSA). This fusion protein is expressed in *Pichia pastoris* and, as is readily apparent from the example, the FcγRII component is produced in a naturally glycosylated form. Page 50, lines 2-11 describes that the molecular weight of the FcγRII component varies according to the expression system, and that this various is due to differences in glycosylation. Plainly, the person skilled in the art would understand that the "fusion component" in this example is *not* the carbohydrate(s) linked to the FcγRII component through the natural glycosylation process, but rather the foreign HSA fusion component at the N-terminal of the FcγRII component.

Applicant submits therefore, that Peltz, et al., does not describe an FcγRII receptor and a fusion component, wherein the fusion component is a carbohydrate, and therefore does not anticipate Claims 74, 75, 77-79 and 81. Reconsideration is respectfully requested.

The Rejection under 35 U.S.C. § 103(a)

The Examiner has rejected Claims 75 and 79 under 35 U.S.C. § 103(a) as being unpatentable over Peltz, et al., in view of Yeh, et al., *Proc. Natl. Acad. Sci. USA* 89:1904-1908 (1992). The Examiner bears the burden of establishing a prima facie case of obviousness (Section 103). In determining obviousness, one must focus on Applicant's invention as a whole. *Symbol Technologies Inc. v. Opticon Inc.*, 19 U.S.P.Q.2d 1241, 1246 (Fed. Cir. 1991). The primary inquiry is:

> whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have had a reasonable likelihood of success . . . . Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure.

In re Dow Chemical, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988). Specifically, the rejection reasons that Yeh, et al., teaches that due to certain characteristics of HSA, the "fusion of

bioactive peptides to HSA is a plausible approach toward the design and recovery of secreted therapeutic HSA derivatives". The rejection further asserts that it would have been obvious to "combine the teachings of Peltz, et al., and Yeh, et al., to arrive at the fusion of soluble FcγRII linked to HSA as taught by Yeh, et al. with a reasonable expectation of success.

Applicant respectfully traverses this rejection. Yeh, et al., exemplifies the expression of a *single* fusion protein comprising a soluble CD4 receptor polypeptide fused to HSA. As explained below, and with the possible exception of proteins closely related to CD4, the person skilled in the art would not have held any "reasonable expectation of success" in applying the teachings of Yeh, et al., in the fusing an FcγRII component (e.g. the soluble extracellular region of FcγRII) to HSA, or any other "fusion component", while maintaining the biological activity of that FcγRII component. Reasonable expectation of success is assessed from the perspective of the person of ordinary skill in the art. *See Micro Chem., Inc. v. Great Plains Chem. Co.*, 103 F.3d 1538, 1547, 41 U.S.P.Q.2D (BNA) 1238, 1245.

The extracellular region of FcγRII is dissimilar to the CD4 receptor investigated by Yeh, et al. The lack of similarity in the extracellular regions FcγRII and the CD4 receptor weighs against a reasonable expectation of success. Additionally, in circumstances where a relatively small protein such as the FcγRII extracellular region (26/30 kD) is to be expressed as a fusion protein with a large protein such as HSA (67 kD), there is little *a priori* predictability as to whether the biological activity of the FcγRII extracellular region will be maintained. This is because there is a likelihood that the HSA fusion component will interfere with the correct folding of the FcγRII component and, additionally, a very real possibility that the fusion component could sterically obscure the immunoglobulin-binding site of the FcγRII component. Neither Yeh et al., or Peltz et al., provide any information or guidance which would have allowed the person skilled in the art to dismiss these risks and thereby reasonably predict the success of expressing a biologically active FcγRII component as a fusion protein comprising a fusion component such as HSA. Furthermore, Yeh, et al., contains only a single example of expression of a fusion protein comprising HSA. Absent any further exemplification of the successful expression of non-CD4 receptor proteins fused to HSA, it is submitted that the person skilled in the art in 1994 (the priority date of the present claims), would have held considerable doubt as to as to the success of such an approach. The person skilled in the art would have viewed the disclosure of Yeh, et al., at best, as an invitation for further experimentation. At

most, the combination of Yeh, et al., and Peltz, et al., make it "obvious to try" to fuse HSA to a non-CD4 receptor protein. It is well settled that "obvious to try" is an improper consideration in an obviousness rejection. *See Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S.P.Q. 81, 91 (Fed. Cir. 1986).

The rejection appears to use hindsight in making the obviousness rejection in that the rejection finds each element of the pending claims in the prior art, and then reasons that it is easy to reassemble these elements into the invention; however "it is impermissible to use the claimed invention as an instruction manual or "template" to piece together the teaching of the prior art so that the claimed invention is rendered obvious." *In re Fritch*, 972 F.2d 1260, 1266, 23 USPQ2d 1780, 1784 (Fed. Cir. 1992). The invention must be viewed not with the blueprint drawn by the inventor, but in the state of the art that existed at the time." *Interconnect Planning Corp. v. Feil*, 774 F2d 1132,1138, 227 USPQ 543,547 (Fed. Cir. 1985).

It was not until the present inventors carried out the experimentation described at Example 6, that they first became aware that the possibility of expressing a biologically active FcγRII component as a fusion protein could be successful. The knowledge gained from that experimentation and, particularly, the realization that a large fusion component such as HSA does not destroy the biological activity of the FcγRII component, subsequently led the inventors to investigate the expression of an FcγRII component with another fusion component, namely the bacterial maltose binding protein (43 kD). As is mentioned at Example 12 of the present specification (see, particularly, page 61, lines 15-22), a fusion protein comprising the extracellular region of FcγRII and bacterial maltose binding protein (denoted WMBPrsFcR) was expressed and was biologically active (i.e. the FcγRII component retained the ability to bind to immune complexes). Taken together, these results allowed the present inventors to predict that an FcγRII component could be successfully expressed as a fusion protein comprising other fusion components such as immunoglobulin, polyethylene glycol and complement receptors, etc. (see the further discussion of other suitable fusion components at, for example, page 52, lines 7-37). Thus, the present inventors (and others) have subsequently expressed a biologically active FcγRII component as a fusion protein comprising the functional domain of the human complement regulatory protein CD46 (see the enclosed copy of Lanteri, M.B. et al., *Transplantation*, 69(6):11281136, Mar 2000).

That the inventors were ultimately successful is irrelevant to whether one of ordinary skill in the art, at the time the invention was made, would have reasonably expected success. *See Standard Oil Co. v. American Cyanamid Co.*, 774 F.2d 448, 454; 227 USPQ 293, 297 (Fed. Cir. 1985). Using the inventors' success as evidence that the success would have been expected is an impermissible use of hindsight. *See In re Kotzab*, 217 F.3d 1365, 1369, 55 U.S.P.Q.2D (BNA) 1313, 1316 (Fed. Cir. 2000).

Given that the combined teachings of Peltz, et al., and Yeh, et al., provide no reasonable expectation of success in the provision of the fusion of soluble FcγRII linked to HSA, Applicant respectfully submits that the combination of Peltz, et al., and Yeh, et al., cannot render obvious Claim 75 or 79. Reconsideration is respectfully requested.

Applicant believes that the pending claims are in condition for allowance. If it would be helpful to obtain favorable consideration of this case, the Examiner is encouraged to call and discuss this case with the undersigned.

Respectfully submitted,

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INHIBITION OF HYPERACUTE TRANSPLANT REJECTION BY SOLUBLE PROTEINS WITH THE FUNCTIONAL DOMAINS OF CD46 AND FcγRII[1] [Immunobiology]

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Abstract TOP

Background. Recombinant soluble forms of complement regulatory molecules, including the human complement regulatory protein CD46 (rsCD46), have been shown to inhibit hyperacute transplant rejection (HAR) and protect against complement- mediated inflammatory tissue damage.Similarly, recombinant soluble forms of the immunoglobulin receptor FcγRII (rsFcγRII) can attenuate antibody-mediated inflammatory responses. We have produced and tested the function of novel recombinant chimeric proteins that incorporate the functional domains of both CD46 (membrane cofactor protein, MCP) and the low affinity human IgG receptor FcγRII (CD32).

Methods. Two recombinant soluble chimeric proteins (CD46:FcR and FcR:CD46) were designed and produced using a human cell expression system. Their ability to protect cells against complement-mediated lysis (through the CD46 domain) and bind human IgG (through the Fc

Article Outline

receptor domain) was assessed in vitro. They were also tested in vivo in the rat reverse passive Arthus reaction and a murine model of hyperacute cardiac transplant rejection.

Results. In vitro, the functional domains of the chimeric proteins each retained their activity. In vivo, the serum half-life of the recombinant chimeric proteins in mice was more than either rsCD46 or rsFcγRII. In the rat reverse passive Arthus reaction, intradermal injection of each recombinant protein substantially reduced inflammatory skin edema (>50%) and polymorphonuclear neutrophil infiltration (>90%). In the hyperacute rejection model, i.v. treatment with FcR:CD46 prevented complement- mediated rejection, macroscopic bruising, edema, and thrombosis more effectively than rsCD46.

Conclusions. CD46/FcγRII bifunctional proteins have an improved ability to control complement- mediated hyperacute graft rejection and have therapeutic potential in other conditions involving antibody-mediated inflammation.

Inflammatory immune responses that involve complement activation, alone or together with antibody in immune complexes, are central to the innate and cognate immune systems. Activation of complement can result in opsonisation, lysis of cells by the membrane attack complex, chemotaxis, and the activation of inflammatory cells. These responses are primarily beneficial, but they can also cause tissue damage in acute inflammation or as part of disease processes. The hyperacute rejection of vascularized xenotransplants, resulting from the activation of complement by naturally occurring antibodies (1), is a spectacular example of such damage.

The complement cascade is regulated to prevent inappropriate activation on cell plasma membranes and within body fluids by a number of cell surface and soluble proteins. Several recombinant soluble complement regulators, including rsCD46 and rsCR1 (complement receptor type 1, CD35), have been shown to protect tissues against damage in a variety of pathologies, and have potential therapeutic applications (2, 3).

Inflammatory responses can also be mediated through Fcγ receptors (FcR). Immune complexes of IgG antibody bound to antigen can interact, through binding of the immunoglobulin Fc portion, with cell-surface Fcγ receptors on many cells of hematopoetic lineage, including monocytes, macrophages, neutrophils, platelets, dendritic cells, and B lymphocytes, resulting in clearance of the complexes or modulation of the immune response (4). Deposition of immune complexes is associated with several diseases, such as rheumatoid arthritis, glomerulonephritis, and systemic lupus erythematosus (5), and Fcγ receptors have been shown to be important in animal models of immune-complex disease (6, 7). Naturally occurring soluble forms of these molecules may provide physiological inhibition of this arm of the immune response (8). Recombinant soluble FcγRII can inhibit inflammation, presumably by blocking immune complex binding to cell-surface Fc receptors (9).

It is possible that chimeric soluble molecules that can both bind immune-complexes and regulate complement might be more effective than molecules possessing either function alone. To investigate this, novel bifunctional molecules were produced that combine the functional modules of the human complement regulator CD46 with the Fcγ-binding activity of FcγRII.

CD46 [membrane cofactor protein (MCP)] is one of the key human proteins for complement regulation that is expressed on all nucleated cells. It is able to irreversibly inactivate complement early in the activation cascade, through cofactor activity for the degradation of C3b and C4b by the serum protease factor I, and is especially efficient at inhibition of the alternative complement pathway (3, 10). Its short consensus repeat (SCR) modules mediate this activity. Recombinant forms of CD46, expressed as cell surface proteins on

transfected cells (11, 12) in transgenic mice (13-15), or as recombinant soluble proteins (rsCD46) (3, 16) are effective in protecting cells against complement-mediated lysis and inhibit complement-mediated tissue damage in vivo by reducing complement deposition. The ability of human CD46 to regulate complement activation using serum complement from different species (including human, pig, rabbit, guinea pig, rat, and mouse) (3, 11, 16) enables its evaluation in a variety of experimental models.

FcγRII has a low affinity for monomeric human immunoglobulin ($K_A < 107\ M^{-1}$) (17). In soluble form it is likely to circulate free in vivo rather than bound to circulating IgG, and should thus be available to localize within sites of inflammation. Moreover, human rsFcγRII can bind immune complexes consisting of either human, rat or mouse IgG (9) and thus can be applied to a variety of experimental models.

Two chimeric proteins were constructed, with different relative orientations of the CD46 and FcγRII components, designated CD46:FcR and FcR:CD46. These and several other control recombinant proteins (depicted in Fig. 1) were tested for the presence of conformation-dependent epitopes, for the ability to regulate complement activation and bind IgG in vitro, and for the ability to modulate inflammatory immune responses in vivo.

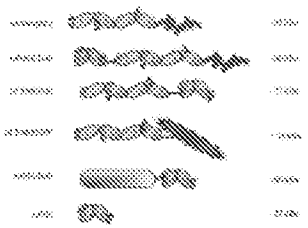


Figure 1. Representation of recombinant soluble chimeric proteins. The modular components of rsCD46 are the □60 amino acid short consensus repeat (SCR) domains, SCR 1-4, which encompass the C3b and C4b binding and cofactor activity functions, and the serine, threonine, proline-rich (STP) BC sequence that is heavily O-glycosylated. The BC sequence was included in chimeric proteins with CD46 at the C-terminus. The rsFcγRII protein contains two domains (D1 and D2) that are members of the Ig super-family. Analysis on SDS-PAGE and Western blotting established the approximate molecular weight. The CD46 SCR 1, 2, and 4 modules and the two FcR domains each have a site for N-linked carbohydrate as indicated.

MATERIALS AND METHODS TOP

Animals. TOP

Inbred mice, Gal o/o (18), C57BL/6J, and (CBAxBALB/c)F1 and outbred rats (Sprague Dawley) were bred or held at the Austin Research Institute.

Cell lines, antibodies, and serum. TOP

CHO-K1 (Chinese hamster ovary fibroblasts; ATCC CCL 61), and 293-EBNA (EBNA transduced human embryonal kidney fibroblasts) (Invitrogen, Carlsbad, CA) were grown in DMEM culture medium (CSL, Melbourne, Australia) supplemented with 10% fetal bovine serum (CSL), 20 μg/ml proline, 14 μg/ml hypoxanthine, and 3.9 μg/ml thymidine. Monoclonal antibodies: E4.3 anti-CD46 (SCR1 module; IgG2a) (19), M177 anti-CD46 (SCR2 module; IgG1) (20), 8.26 anti-FcγRII (IgG2b), and 8.7 anti-FcγRII (IgG1) (21) were immunopurified on protein A-Sepharose. Human serum from a single donor was freshly prepared and held at 4°C for immediate use in the heart transplant model, or was aliquoted and stored at -70°C for cell lysis assays.

Construction of chimeric CD46:FcγRII and FcγRII:CD46 proteins. TOP

Expression constructs were derived from cDNA templates previously used to generate recombinant soluble proteins: rsCD46(BC) (16) and human rsFcγRIIa-H131 (21). The splice-overlap-extension polymerase chain reaction (PCR) method was used to generate individual fragments which in the final PCR produced chimeric constructs ready for ligation into the APEX-3 vector (22). The CD46 and FcγRII fragments were joined by a 15-bp linker encoding Gly-Gly-Gly-Lys-Gly. PCR primers were designed to anneal to the 5′-coding sequence and designated 3′-ends of each template and to encode the linker sequence, and anneal, respectively, to the 5′- and 3′-ends of the sequences required at the junctions of the functional domains. Primer sequences, 5′ to 3′, were for FcR:CD46:

P1 (5′-FcR) GAATTCCAACTATGGAGACCCAAATGTCTCAG: P2 (linker-3′-FcR):

CTCACAGGCACCCTTGCCTCCACCTTGGACAGTGATGGTCACAGGC: P3 (linker:5′-CD46): ACTGTCCAAGGTGGAGGCAAGGGTGCCTGTGAGGAGCCACCAACATTTG: and P4 (3′-CD46) ACGAATTCGATTTCAAGCCACTTTCTTTACAAAG); and for CD46:FcR: P5 (5′-CD46) CGGAATTCACAGCGTCTTCCGCGCCGCGC: P6 (linker:3′-CD46): TGGGGGAGCACCCTTGCCTCCACCCACTTTAAGACACTTTGGAACTGG: P7 (linker:5′-FcR): CTTAAAGTGGGTG GAGGCAAGGGTGCTCCCCCAAAGGCTGTGCTGAAAC: and P8 (3′-FcR) TCGCCCGATTCTAGACTATTGGACAGTGATGGTCAC. Full cDNA sequences are accessible as GenBank M58050 (CD46) (23) and GenBank J03619 (FcγRIIA) (24). All constructs were fully sequenced to ensure they were free of errors. The rsCD46:FcR construct consists of the first 6 exons of CD46 (encoding the leader peptide and four SCR modules), then the 5 aa linker, and cDNA encoding the two Ig-superfamily extracellular domains of FcγRII. For rsFcR:CD46 the order of functional domains is reversed and in addition contains CD46 exons 8 to 10, encoding the 46 residue O-glycosylated membrane-proximal sequence designated STP-BC at the carboxy terminus. Two other recombinant constructs, encoding rsCD46:EGFP and HSA:FcR, were used to make control proteins. The rsCD46:EGFP protein was expressed and purified similarly to the bifunctional chimeras, from a cDNA construct provided by S. Russell and O. Yuriev (The Austin Research Institute) that encodes the four SCR modules of CD46 followed by the enhanced green fluorescent protein sequence (25). HSA:FcR consists of human serum albumin sequence at the amino terminus followed by the two FcγRII extracellular domains (Powell M, manuscript in preparation). Figure 1 shows diagrammatically the structures of the chimeric proteins.

Production and purification of recombinant proteins. TOP

Expression constructs were transfected into 293-EBNA cells and stable clones were selected with 100 μg/ml hygromycin. Production of recombinant CD46 protein was detected by radioimmunoassay (RIA) using anti-CD46 monoclonal antibodies. Supernatants from transfected cells grown in serum-free medium were passed through a CD46 affinity column containing monoclonal antibodies E4.3, eluted with 25 mM diethylamine pH 11.4, dialyzed against phosphate-buffered saline, and concentrated using Centricon tubes (Amicon, Bedford, MA) (16). Affinity columns of heat-aggregated human gammaglobulin were used to purify rsFcγRII and HSA:FcγRII (9). Concentrations were determined by measuring spectroscopic absorbance at 280 nm (26).

Protein assays. TOP

The purified CD46 chimeric proteins were analyzed by one- and two-dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by silver staining, or electroblotting to filters and Western blotting with anti-CD46 monoclonal (E4.3) antibody. Radioimmunoassays utilized the anti-CD46 (E4.3) or anti-FcR (8.26) plate-bound monoclonal antibodies to capture protein and iodinated anti-CD46 M177 monoclonal antibody as tracer (3). Standard curves were prepared from purified proteins. A similar protein capture protocol with an ELISA readout used detection by the horse-radish peroxidase (HRP)-conjugated anti-FcR monoclonal antibody 8.7. Binding of recombinant proteins to human IgG1, IgG3, and IgE was measured using a BIAcore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden).

Biodistribution in mice. TOP

The chimeric rsCD46:FcR and rsFcR:CD46 proteins were iodinated using the chloramine T method. Mice [12-week-old (CBAxBALB/c)F1 males and females] were injected i.v. with 600,000 cpm protein (□11-15 ng) diluted to 0.2 ml in PBS. Blood samples were taken at measured intervals and mice were killed at specific times to sample tissues, which were weighed immediately, to measure 125-Iodine content.

Complement-mediated lysis. TOP

In a hemolysis assay, washed sheep erythrocytes coated with rabbit anti-sheep Ig were aliquoted (0.10 ml of 1% v/v), mixed with human serum as complement source (to a final concentration of 0.5%) and recombinant protein to a final volume of 0.20 ml, and incubated for 30 min at 37°C. Hemolysis in the supernatants was measured by absorbance at 405 nm. In a cell lysis assay, CHO-K1 cells, 30,000 per replicate wells, were incubated with polyclonal rabbit anti-CHO serum (final dilution 1/100), rabbit or human serum (as complement source), and recombinant proteins, in 0.05 ml for 60 min at 37°C. The reaction was stopped with 0.15 ml cold PBS, and cell viability was counted by aniline blue dye exclusion (3).

Reverse passive arthus reaction in rats. TOP

Anesthetised female rats (120-150 g) received injections i.v. with 0.6 ml of PBS containing 5 mg ovalbumin and 2% w/v Evans Blue dye, shaved along the back and injected intradermally with 0.10 ml of PBS containing 0.5 mg purified rabbit-anti-OVA IgG with or without recombinant soluble proteins, or PBS alone. The same dose (58 μg) of each chimeric recombinant protein was given. Mixtures of rsCD46 and rsFcγRII were also tested at two doses; 29 μg of each and 58 μg of each. The rats were killed after 6 hr, and the area of dye leakage at each injection site, representing edema, was measured. Skin samples were dissected, fixed with formalin, and hematoxylin and eosin stained parrafin-block sections were inspected microscopically for edema and cellular infiltration.

Heart transplant model in mice. TOP

Hearts from 8- to 12-week-old C57BL/6 mice were grafted heterotopically into the abdomen of anesthetized 12-week-old Gal o/o recipients, reperfused, and observed continuously until rejection occurred or for 120 min (1). To precipitate rejection, 0.5 ml of freshly prepared human serum (from one donor) was injected slowly into the recipient's inferior vena cava. The recombinant soluble proteins were injected into the inferior vena cava 10 min prior to the serum, as equimolar doses of 4.5 nM, corresponding to 0.25 mg of rsCD46, 0.40 mg of HSA:FcR and FcR:CD46, and 0.35 mg of CD46:EGFP. Because a limited number of Gal o/o mice were available, the CD46:FcR construct was not used. In control experiments, PBS was injected instead of recombinant protein. Experiments were terminated and grafts taken for histology after rejection (the complete loss of contractile activity) or after 90 min.

Statistical analysis. TOP

P values were calculated for continuous variables using Student's *t* test and for proportions by applying a $\chi 2$ test to the relevant 2×2 table.

RESULTS TOP

Purified recombinant chimeric proteins express conformationally dependent epitopes. TOP

Each recombinant protein was tested for homogeneity by SDS-PAGE analysis on single and two-dimensional gels, followed by silver staining. For each, a single predominant protein species was detected with minor bands sometimes observed after overdevelopment, at sizes corresponding to nonglycosylated protein (Fig. 2 A). The observed protein sizes were consistent with the predicted full length mature glycosylated proteins as confirmed by Western blotting using several monoclonal and polyclonal antibodies. Thus, rsCD46:FcR was □75 kDa (3 N-glycosylation sites on CD46 plus 2 on FcγRII; see Fig. 1), rsFcR:CD46 was □90 kDa (with multiple O-glycosylation sites on the CD46-BC sequence), and rsCD46 was □55 kDa. Staining with the functionally-blocking conformation-dependent monoclonal antibodies E4.3 (Fig. 2B) or M177 (data not shown) to detect CD46, or 8.26 (anti-FcR) (data not shown), indicated that the functional domains of the secreted proteins had retained their native tertiary structure, and the positions of these aligned with the proteins detected by silver staining.

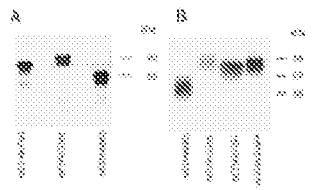


Figure 2. SDS-PAGE analyses of the recombinant proteins. Recombinant proteins were run on non-reducing gels and either silver-stained (A) or electroblotted and probed with anti-CD46 mAb E4.3 (B). Proteins loaded for silver staining had been immunopurified, whereas Western blotting was of 10-μl samples of supernatant from stably transfected cell lines. Molecular weight standards were used to estimate sizes of the recombinant proteins.

RIA and ELISA assays, in which the capture and probe reagents reacted with the different components of the chimeras, were used to further quantitate the recombinant proteins and demonstrate that multiple conformation-dependent and functionally important epitopes were present. Thus, plate-bound anti-FcR mAb 8.26 was used with iodinated-M177 anti-CD46 (Fig. 3) or HRP-conjugated E4.3; and plate-bound anti-CD46 mAb E4.3 was used with anti-FcR HRP-conjugated monoclonal antibody 8.7 (data not shown). These

assays demonstrated that the epitopes were each present on all of the secreted recombinant molecules. Comparison with standard curves gave estimated concentrations of the chimeric proteins accumulating in culture supernatants to approximately 5 µg/ml. BIAcore analysis demonstrated that the FcR component of the recombinant chimeric proteins bound specifically to human IgG1 and IgG3, but not IgE, similar to rsFcγRII (data not shown).

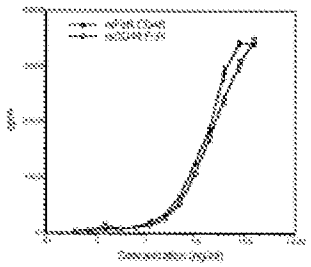


Figure 3. Binding of chimeric rsFcR:CD46 and rsCD46:FcR proteins to conformation-dependent monoclonal antibodies. Immunopurified proteins were analyzed by radioimmunoassay, using plate-bound anti-FcR mAb 8.26 as capture monoclonal antibodies and detection by iodinated-M177 anti-CD46. Concentrations of the recombinant proteins were determined by absorbance. The data show the mean of duplicate samples (±SD) and are typical of multiple assays.

CD46 complement regulation is maintained in both chimeric proteins. TOP

Two assays of complement-mediated cell lysis demonstrated that both rsCD46:FcR and rsFcR:CD46 proteins protected cells. Half-maximal lysis of sensitized erythrocytes by 0.5% human serum (C') was achieved with 125 nM rsCD46:FcR and rsFcR:CD46 (□10 µg/ml), and maximal inhibition occurred with >1 µM (□80 µg/ml) of either protein (Fig. 4 A). Lysis of CHO-K1 cells (detected by aniline blue dye uptake) required higher human serum (C') concentrations, and higher concentrations of these recombinant proteins were required for inhibition (Fig. 4B). Rabbit serum (C') produced lysis at lower concentrations, and the recombinant proteins were highly effective at protection (Fig. 4C). The rsCD46:EGFP was as effective as the FcR/CD46 chimeras, demonstrating that the C-terminal globular EGFP molecule had no steric effects on CD46 function (Fig. 4C), although rsFcγRII had no protective activity (data not shown). Titration of each CD46-containing recombinant protein generated similar inhibition curves, also demonstrating that the FcR component did not interfere with binding of C3b or cofactor activity mediated by the CD46 SCR modules.

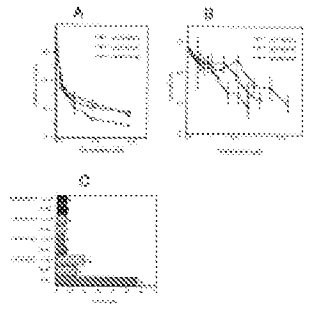
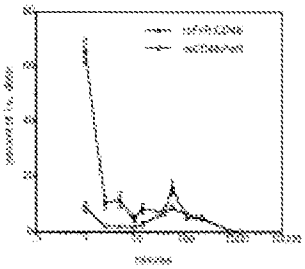


Figure 4. Lysis of erythrocytes and CHO-K1 cells is regulated by the chimeric proteins. Hemolysis of sensitized erythrocytes occurred in the presence of 0.5% human serum and titrated chimeric proteins (A). CHO cell lysis was measured as aniline blue dye uptake into CHO-K1 cells in the presence of 20% human serum with titrated proteins (B), or the presence of 3% rabbit serum at two protein concentrations (5 and 10 µM) (C). The data show the means±SD of triplicate wells and are representative of independent experiments.

Biodistribution of recombinant proteins in mice. TOP

The chimeric proteins were rapidly cleared from the blood after i.v. injection (Fig. 5): the estimated alpha-phase half-life for rsFcR:CD46 was 1.3 min, and for rsCD46:FcR was approximately 20 sec. The comparable alpha-phase half-life of rsCD46 has been previously calculated as less than 1 min (3). The initial rapid removal of protein from the circulation was associated with uptake of Iodine-125 into the liver (data not shown), then a gradual release back into the circulation peaking at about 1 hr. Circulating recombinant protein was readily detectable by radioimmunoassay 90 min after injection of 0.25 mg rsCD46 or 0.4 mg rsFcR:CD46, at an approximate concentration of 0.6 µg/ml (data not shown), suggesting that protein remaining after the first few minutes was cleared more slowly.

Figure 5. Blood content and clearance of chimeric proteins after i.v. injection. Blood samples taken from five mice receiving injections with radiolabeled chimeric proteins were weighed, the radioactive content was determined, and



expressed as a mean percentage of the injected dose assuming that the total blood volume was □7% of total body mass.

Reduction of inflammation in the reverse passive arthus response in rats. TOP

Clearly delineated skin lesions, mean area □250 mm (2), developed at the sites of anti-OVA Ig intradermal injection in rats given ovalbumin i.v. (Fig. 6). A significant reduction (>50%;*P* <0.05) in the area of edema was seen when the recombinant proteins (58 μg) were injected into the dermis with antibody and all had similar inhibitory effects. Thus, smaller lesions occurred in the presence of either rsFcR, rsCD46, or the chimeric proteins, and mixing rsFcR with rsCD46 was not more effective (Fig. 6). Microscopic examination of sites injected with anti-OVA Ig alone revealed a generalized and vigorous cellular infiltrate within the dermis, predominantly of polymorphonuclear cells, with perivascular cuffing (Fig. 7, A and B). Injection of recombinant protein or PBS buffer alone resulted in a largely normal histological picture (Fig. 7C). Addition of the recombinant proteins to the anti-OVA Ig caused a markedly reduced infiltrate throughout the injection site (rsFcR:CD46 shown in Fig. 7D) with the number of PMN scored being <10% of the normal Arthus reaction. Thus, both CD46 and FcγRII modules inhibited the cellular infiltrate induced by immune complexes and complement activation in the Arthus lesions.

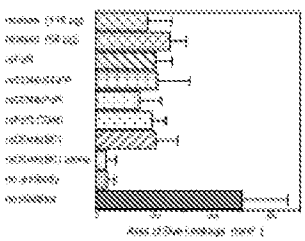


Figure 6. Reduction in reverse passive arthus edema mediated by the recombinant proteins. Affected skin sites (n=5), stained by i.v. injected Evans Blue dye, were measured and the mean area (±SD) was calculated. Negative control skin sites were injected with no antibody or recombinant protein (rsCD46) alone; all other sites received anti-OVA antibody with or without recombinant protein.

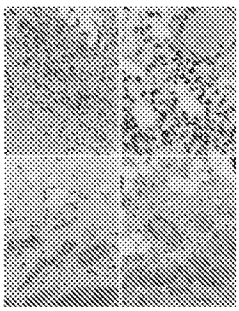


Figure 7. Reduction in reverse passive arthus cellular infiltrate mediated by the recombinant proteins. Hematoxylin and eosin-stained paraffin embedded skin sections were prepared from skin sites injected with anti-OVA antibody in the absence of recombinant proteins (A, x20; B, x50), and from sites injected with recombinant protein only (rsCD46 in C, x20) or with anti-OVA antibody plus recombinant protein (D, x20 shows the effect of rsFcR:CD46).

Chimeric proteins prevent hyperacute rejection of mouse heart allografts. TOP

The allotransplantation of mouse hearts provides a sensitive model of vascularized organ grafts that have been subjected to a period of ischemia: the addition of human anti-Gal antibodies and complement is sufficient to cause hyperacute rejection (1). All Gal+ mouse hearts grafted into Gal o/o mice and given 0.5 ml human serum, but no recombinant protein, were hyperacutely rejected (mean time to rejection 35 min; n=7) (Table 1). Rejection was manifested macroscopically by graft swelling, cyanosis, and loss of contractile function. Histology revealed vascular occlusion and sporadic disruption of the endothelium but no interstitial

hemorrhage (Fig. 8 A). Treatment with HSA:FcR had no effect: all four hearts were rejected with a mean time of 28 min (P =0.2 compared with no treatment) (Table 1). Treatment with the bifunctional chimeric protein FcR:CD46 at a dose of 4.5 nM (0.4 mg) completely prevented hyperacute transplant rejection (n=5). The macroscopic and microscopic appearances of these grafts, including heartbeat, were normal throughout the 90-min observation period (Fig. 8B). Treatment of five transplants with equimolar doses of rsCD46 gave variable results. Three hearts were rejected (mean time to rejection 33 min) with histological changes typical of HAR, and two hearts survived more than 90 min (when the experiment was terminated). These nonrejected hearts displayed transient macroscopic swelling and cyanosis within the first 30 min after serum injection, although this was not as pronounced as seen in the hearts that progressed to rejection, and histological examination revealed evidence of edema and tissue damage without occlusive thrombosis (Fig. 8C). A final group of transplants was treated with rsCD46:EGFP. None of these grafts were rejected within the 90-min observation period, although three displayed macroscopic swelling and cyanosis and a microscopic appearance similar to Figure 8C.

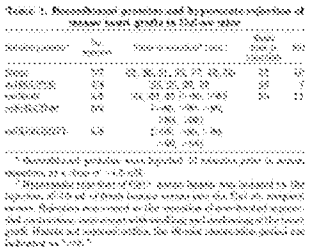


Table 1. Recombinant proteins and hyperacute rejection of mouse heart grafts in Gal mice[a] Recombinant proteins were injected 10 minutes prior to serum injection, at a d □4.5 nM.[b] Hyperacute rejection of Gal+ mouse hearts was induced by the injection o of fresh human serum into the Gal o/o recipient mouse. Rejection was scored as the cessation of coordinated myocardial contraction, concurrent with swelling and darken the heart graft. Hearts not rejected within the 90-min observation period are indicated

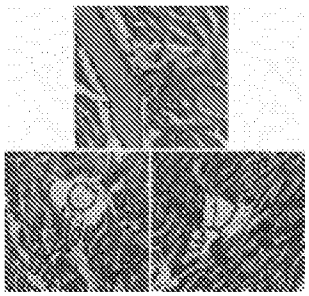


Figure 8. Histology of transplanted mouse hearts showing hyperacute graft rejection or protection by chimeric protein. Hematoxylin and eosin-stained sections x20. Transplanted hearts were taken after complete hyperacute rejection had occurred (defined by cessation of contractions) after injection of human serum (A) or when still functioning 90 min after the separate injections of human serum and a single preceding 4.5 nM dose of rsFcR:CD46 (B) or rsCD46 (C).

DISCUSSION TOP

In this study we report the design and production of two novel recombinant soluble chimeric proteins that combine the functional domains of the complement regulator CD46 and the human IgG-binding low affinity Fc receptor (FcγRII). Previously, we had studied the effects of recombinant soluble CD46 (rsCD46) and rsFcγRII alone in the reverse passive Arthus reaction, and noted that although they were each highly inhibitory [at maximal doses of 70 μg for rsCD46 (3) and 500 μg for rsFcγRII (9)], they were unable to entirely block the inflammation. Similarly, rsCD46 delayed hyperacute graft rejection in a rodent model, reducing the early deposition of complement but not preventing organ destruction (3). It was possible that both rsCD46 and rsFcγRII together would be more efficient, and that combining the two functional domains in a novel protein might result in enhanced antiinflammatory function. First, Ig-binding properties could serve to concentrate such proteins on surfaces coated with immunoglobulin or immune complexes. Second, the positioning of a complement regulatory domain close to an immunoglobulin molecule could enhance its ability to regulate the classical pathway, which is activated by antibody. Third, an increase in the molecular weight afforded by the addition of functional modules could result in an increase in a longer half-life (a known limitation of proteins <60 kDa; the molecular weight of rsFcγRII is 25 kDa and of rsCD46 is 55 kDa).

The two chimeric proteins, with alternate positioning of the components, were shown to conserve conformation-dependent epitopes and to possess the functional attributes of rsCD46 and rsFcγRII. Both chimeric proteins functioned similarly in vitro; there were no obvious effects resulting from the different orientations of the functional domains or from steric effects of adjacent modules. The dose-effect curves presented here for protection of CHO-K1 cells from lysis by rsCD46 (Fig. 4B) closely paralleled those of a

previous study in which 1-2 μM concentrations were found to be effective (16).

The chimeric proteins retained the in vivo ability of both parent molecules to inhibit the reverse passive Arthus reaction in rats. The demonstration by microscopy of a markedly reduced cellular infiltrate (PMN <10% of maximum) was a more sensitive indicator of antiinflammatory activity than the reduction of edema as determined by dye leakage. No added inhibition was seen for the chimeric molecules, or for mixtures of both parent molecules; this suggests that either higher concentrations of the inhibitors are needed, or that inflammatory processes additional to complement activation and Fc receptor interactions are involved.

The development of experimental model systems to study hyperacute organ graft rejection, as occurs for discordant xenografts such as pig organs into humans, can be complicated. The recent production of mice in which the α1,3 galactosyl transferase gene has been inactivated by homologous recombination (Gal o/o) (18) has provided a more elegant system and one closer to xenograft combinations likely to be used clinically. Heart allografts transplanted from normal (Gal+) inbred mice to the abdominal cavity of Gal o/o recipients are rejected in about a week by cell-mediated responses, unless the recipients are hyperimmunized and have a high titer of anti-Gal antibodies, or anti-Gal antibody plus complement (e.g., fresh human serum) is injected: then hyperacute rejection occurs, with fibrillation and bruising evident within 10 to 15 min, leading to thrombosis, engorgement of the graft, and rapid loss of myocardial contraction (1).

The chimeric molecule rsFcR:CD46 prevented hyperacute heart graft rejection more reliably than equivalent molar amounts of rsCD46 (P <0.05). This process is predominantly mediated by naturally occurring anti-Gal IgM antibodies in human serum, although deposition of IgG antibodies (which are not capable of causing HAR alone) also occurs (1). The observation that soluble forms of FcγRII did not influence the process is to be expected. The enhanced activity of rsFcR:CD46 over rsCD46 suggested that the FcγRII domain may be providing a useful function, possibly by allowing the bifunctional protein to localize to the areas of immunoglobulin deposition, or through contribution to protein size. The circulating half-life in mice of rsFcR:CD46 (alpha-phase 1.3 min, with an extended beta-phase), which is more than that of rsCD46 (alpha-phase <1 min) (3), may explain its increased efficacy. In support of this explanation, another recombinant construct with an increased molecular weight, rsCD46:EGFP, was also better able to inhibit HAR in the mouse model than rsCD46. However, the ability of FcR:CD46 to prevent much of the macroscopic and histological evidence of tissue damage, which was still apparent in animals treated with rsCD46:EGFP, suggests that the addition of an FcγRII domain may be useful apart from any increase in half-life.

The in vivo rodent models of inflammation and disease are convenient experimental models to establish principles of function; however, they can suffer from limitations due to different molecular functions between species. FcγRII binds mouse IgG similarly to human IgG, but CD46 functions, involving binding of C3b or C4b and an interaction with the serum protease factor I, appear to be less efficient with rodent serum. We had previously studied the ability of CD46 to inhibit mouse spleen cell lysis mediated by rat complement in vitro and found that it controlled complement activation through the alternative pathway (although not as efficiently as with human complement), but protection against the classical pathway was poor (13). In both in vivo models used in our study, the classical pathway would be expected to play a principal role in inflammation. Thus, the reverse passive Arthus results, involving only rodent complement, probably underestimate the ability of CD46 domains to function in a human clinical setting. In the mouse heart graft model, human serum was injected to precipitate graft rejection. As the relevant human anti-Gal antibodies would be focused almost entirely on the graft tissue in the Gal o/o mice, and the circulating human complement components would tend to be activated at this site, this model provides good evidence of highly effective protection mediated by CD46. These data showed a greater protective effect of CD46 than earlier rodent xenotransplant studies in which the hyperacute rejection of mouse hearts in rats was initiated by immune rat serum (3, 13).

The organs of transgenic animals expressing cell-surface complement regulators (CD46, CD55, and CD59) have been shown to be protected against hyperacute rejection in many studies (2, 13). Soluble CR1 (sCR1) has also been shown to delay hyperacute rejection in small animal models (27) and the pig to primate combination (28). Doses of 15 mg/kg were used to achieve inhibition; the dose of rsCD46 used in this study was 8 mg/kg, although the dose of FcR:CD46 was 13 mg/kg. Inhibition of the reverse passive Arthus reaction in rodents has been demonstrated for several other soluble complement regulators as well as rsCD46; sCR1 (29), soluble CD55 (seDAF) (30), and CAB-2, a soluble complement regulator combining CD46 and CD55 domains (31). All were capable of □50% reduction in edema at intradermal doses ranging

from 10 μg (sCR1) to 100 μg (seDAF). Mast cell degranulation (32) and white blood cell activation through Fc receptors are probably responsible for generation of the remaining edema.

The ability of the CD46/FcR recombinant chimeric proteins to control complement or immune-complex mediated inflammation and hyperacute graft rejection has been clearly demonstrated and we conclude that they have a therapeutic potential, especially where alternative pathway complement activation occurs, and may be superior to soluble complement regulators lacking Fcγ receptor activity. Furthermore, having demonstrated the principle of function, it should not be difficult to add FcγRII domains to other soluble complement regulators, including those able to regulate the antibody-mediated classical activation pathway through other mechanisms (such as CD35 or CD55). This approach may offer a way of improving the antiinflammatory activity of soluble complement regulators in antibody-mediated disease.

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